CENTRAL CO_2 CHEMOSENSITIVITY IN TADPOLES:

IMPAIRMENT AND THE ROLE OF SEROTONIN

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A

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ABSTRACT

Nicotine and ethanol are known neuroteratogens and prenatal exposure correlates with Sudden Infant Death Syndrome (SIDS), which is thought to result from failure to maintain pH homeostasis through respiratory adjustments. This failed homeostatic control is believed to be serotonergic in origin. In previous studies nicotine or ethanol exposure ablated the robust hypercapnic response of early-stage tadpoles. These findings lead us to question if the ablation occurred through a serotonin-dependent mechanism. This study investigated the role of serotonin (5-HT) in the nicotine- or ethanol-induced abolishment of the hypercapnic response. We found that toxin-exposed animals were insensitive to hypercapnia and also failed to respond to concomitant exposure to hypercapnia and 8-OH-DPAT, supporting our hypothesis that toxin-induced abolishment of the hypercapnic response is mediated by 5-HT_{1A} receptors. Immunofluorescence data from brainstem slices of ethanol-exposed animals showed a decrease in 5-HT_{1A} receptors and the serotonin-synthesizing enzyme tryptophan hydroxylase. In contrast, 3-wk nicotine-exposed animals displayed no significant difference in immunofluorescence for either protein. Taken together the electrophysiological and immunofluorescence data suggest the effects of

ethanol or nicotine exposure, which impair the hypercapnic response, include a failure of serotonergic signaling and that this failure is not simply the reflection of a global reduction in serotonin levels.

TABLE OF CONTENTS

LIST OF FIGURES

Page

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GENERAL INTRODUCTION

Introduction

Breathing is vital to life and must be fully functional at birth to orchestrate the first breath or death will surely result (Greer et al., 2007). Breathing's vital roles are to balance the exchange of gases required for the sustainment of life, namely CO_2 and O_2, and also regulate systemic pH balance. There are several pathological conditions in which the control of breathing falters in early development, leading to severe illness and even death (Abu-Shaweesh and Martin, 2008; Chen and Keens, 2004; Gaultier, 2000, 2001; Gozal, 2004).

Ethanol and nicotine are both neuroteratogens and exposure to either during development has been correlated with numerous functional and neurological deficits. Epidemiological studies have determined that infants born to mothers who use nicotine and/or ethanol during pregnancy have an increased risk of Sudden Infant Death Syndrome (SIDS), sparking interest into research regarding the possible mechanistic causes of SIDS (Blair et al., 2006; Iyasu et al., 2002; Kinney et al., 2003; Kinney, 2009; Milerad and Sundell, 1993). SIDS is the result of a central neurological deficit, and one wonders if ethanol and nicotine contribute to SIDS by inducing this deficit.

Sudden Infant Death Syndrome and Homeostatic Impairment

Unlike most medical conditions, SIDS is a diagnosis of exclusion. If an infant under 1 year of age dies and all other causes of death are ruled out, then the death is determined to be SIDS (Willinger et al., 1991). This definition creates an interesting situation insomuch as identifying a cause of a subset of SIDS deaths would then, by definition, exclude those deaths from being diagnosed as SIDS. Therefore to eliminate SIDS entirely, the cause of all infant deaths needs to be ascribed. The ultimate goal is to eliminate these deaths entirely and the first step of this is determining their cause so that preventative measures or treatments can be developed.

In SIDS deaths, the lack of an otherwise identifiable cause of death suggests that SIDS infants simply may have difficulty maintaining physiological homeostasis and that physiological stressors readily handled by normal infants become life-threatening for the SIDS infant (Filiano and Kinney, 1994; Harper et al., 2000; Kinney, 2009). When faced with a physiological stressor an infant can compensate, adapt, or succumb to the loss of homeostasis. During sleep, an infant's breathing response to changes in systemic gas conditions are attenuated (Kuwaki et al., 2008). Previous studies have shown that SIDS deaths often occur while an infant is asleep (Harper et al., 2000; Hauck et al., 2003; Hunt, 1989; Kahn et al., 2003) and that prone sleeping infants have a three to

five times greater risk for SIDS than back sleepers (Duncan et al., 2009; Willinger et al., 1991). These observations suggest that an infant's position and state of arousal are relevant to SIDS. Physiological stressors such as hypoxia and hypercapnia can easily occur in a sleeping infant, particularly while prone, and if the infant is unable to appropriately respond, then a SIDS death may result. This leads to the notion that the pathologies of SIDS are impairments of basic homeostatic processes such as detection and management of pH homeostasis.

SIDS and Serotonin

Previous investigators have identified abnormalities in the medullary serotonergic system of SIDS infants (Filiano and Kinney, 1994; Kinney et al., 1983; Kinney et al., 2001; Paterson et al., 2006). These abnormalities include reduced binding of 5-HT_{1A} receptors, a type of serotonin (5-HT) receptor that functions as an autoreceptor in the medullary raphe (Kinney et al., 2001, 2005; Paterson et al., 2006). 5-HT_{1A} receptors are thought to play a role in central hypercapnic sensitivity and in compensatory responses to systemic CO_2 levels (Corcoran et al., 2009; Severson et al., 2003). Additionally, the medullary serotonergic system plays a role in cardiorespiratory activity and arousal, with impairments to this system being exacerbated during sleep or by the prone position during sleep state. While evidence exists

that sleep state plays a biological role in these deficits, the role of sleeping position is generally considered to be one of physically exacerbating the exogenous stressor that the impaired serotonergic system could otherwise handle. To date the impaired cardiorespiratory control hypothesis is considered one of the most compelling explanations for SIDS (Duncan et al., 2010; Hunt, 1992).

An infant with a vulnerability, such as an impaired medullary serotonergic system, that is exposed to hypercapnia at a time when responses to physiological stressors are blunted, like during sleep, may not respond appropriately and death my result. Such a line of thinking has led to the development of the Triple Risk Hypothesis for SIDS. The Triple Risk Hypothesis proposes that SIDS results when an infant with an underlying vulnerability is unable to adequately respond to an exogenous stressor during a critical period of development (Filiano and Kinney, 1994).

Ethanol and Nicotine are Neuroteratogens

Ethanol and nicotine are both neuroteratogens; effects of their use during pregnancy have been well documented and include: increased chance of miscarriage, growth retardation, impaired neural development, and premature birth (Ernst et al., 2001; Haustein, 1999; Muneoka et al., 2001; Olney et al., 2002; West and Goodlett, 1990). While the effects of

nicotine and ethanol exposure are distinct they both display far-reaching effects on neurogenesis and neural signaling (Abreu-Villaça et al., 2003; Aloe, 2006; Mooney and Miller, 2010; Powrozek et al., 2004; Slikker et al., 2005; Slotkin, 2004). Furthermore, while both may contribute to SIDS, the composite functional result of their exposure is dissimilar, and the mechanisms by which they alter fetal development vary.

Ethanol has broad neurological effects on cell replication, neuroendocrine activity, ligand-gated ion channel signaling, and neuronal migration (Camarillo and Miranda, 2008; Gressens et al., 1992; Lee et al., 2008; Weinberg et al., 2008; Zhang et al., 2005). At the synaptic level, ethanol is a GABAmimetic and allosterically potentiates $GABA_A$ receptors (Aguayo et al., 2002; Breese et al., 2006; Criswell and Breese, 2005; Wallner and Olsen, 2008). Furthermore, ethanol reduces neural activity by decreasing NMDA-type glutamate receptor activity (Hoffman et al., 1990; Ron, 2004; Wirkner et al., 1999). The effects of chronic ethanol exposure are generally associated with these inhibitory actions (Harris and Mihic, 2004), yet chronic ethanol exposure can cause sensitization or desensitization of various neurotransmitter systems (Dopico and Lovinger, 2009; Dubois et al., 2006; Faingold et al., 1998; Trujillo and Akil, 1995).

Nicotine is a nicotinic acetylcholine receptor (nAchR) agonist. nAchRs are widespread components of the cholinergic signaling system

in both central and peripheral nervous systems (Kinney et al., 1993; Slotkin, 2004) serving vital roles in development as early as gastrulation (Lauder and Schambra, 1999). The cholinergic system aids in mediation of neuronal development during the transition from primary replication to terminal differentiation (Slotkin, 1998; Slotkin et al., 2007) as well as modulating synaptogenesis and axonogenesis (Slikker et al., 2005; Slotkin, 2004). Chronic stimulation of nAchRs by nicotine can alter any of these processes or result in desensitization, leading to a diminishment of normal cholinergic activity (Gentry and Lukas, 2002; Quick and Lester, 2002). Nicotine exposure also alters serotonergic neuron density and receptor subtype expression (Duncan et al., 2009; Mihailescu et al., 2002; Slotkin et al., 2007). Thus, while nicotine is an agonist of one specific receptor type, its chronic influence is widespread and involves other neural signaling systems.

Effects of Ethanol or Nicotine Exposure on Breathing

Acute ethanol exposure suppresses fetal breathing movements (Brien and Smith, 1991) and chronic prenatal ethanol exposure depresses respiratory responses to perturbations in blood-gas levels (Dubois et al., 2006, 2008a, 2008b). Ethanol's effects on human fetal breathing movements are so great that it can cause a near cessation (Brien and Smith, 1991; McLeod et al., 1983). GABAergic signaling is

fundamental to respiratory rhythmogenesis and is the major target of ethanol exposure (Bonham, 1995; Brockhaus and Ballanyi, 1998; Ren and Greer, 2006). Not surprisingly, both ethanol and GABA decrease breathing activity (Dubois et al., 2006; Gibson and Berger, 2000) Additionally, GABA is implicated in central responses to hypercapnia and hypoxia (Curran et al., 2001; Fournier et al., 2007; Richter et al., 1999). Physiological responses to hypercapnia are believed to be produced in part through reciprocal inhibition of GABA activity and increased 5-HT excitation (Gourine and Spyer, 2001; Horn and Waldrop, 1994; Kuribayashi et al., 2008; Peano et al., 1992).

nAchRs are widespread in regions associated with breathing control (Dominguez Del Toro et al., 1994; Kinney et al., 1993; Wada et al., 1989) and their activation is fundamental to respiratory rhythm generation (Shao and Feldman, 2001; Shao et al., 2008). The neuroventilatory effect of nicotine exposure in the brain varies based on the duration, developmental timing, and concentration of exposure, as well as the research preparation used to test the effect. Chronic nicotine exposure early in development impairs both central and whole-animal ventilatory responses to hypercapnia and hypoxia (Eugenín et al., 2008; Hafström et al., 2005; Milerad et al., 1995; Neff et al., 2004; Simakajornboon et al., 2004).

The Bullfrog as an Experimental Model

The developing bullfrog tadpole model readily mitigates many of the difficulties posed by using a mammalian model. Mammalian fetal development occurs within the maternal organism, which poses difficulties in controlling levels of neuroteratogen exposure and requires an accounting of maternal metabolism and maternal/fetal interactions. For instance, rats metabolize nicotine faster than humans (Hafström et al., 2005). Thus, to reach comparable levels of exposure to fetal rats, a higher dose of nicotine must be used, which could lead to toxic concentrations in the dam and unintended maternal/fetal interactions. In contrast, bullfrogs are oviparous with development occurring via free-living aquatic stages. Developing tadpoles can be housed in aquaria with easily monitored and controlled levels of neuroteratogens (Hedin and Larsson, 1986; Horimoto and Koyama, 1982; Yorio and Bentley, 1976), and both nicotine and ethanol readily cross epithelial layers of bullfrog skin.

The mechanics of bullfrog ventilation and the central control of breathing in bullfrogs are well documented (Gargaglioni and Milsom, 2007; Gdovin et al., 1999; Torgerson et al., 2001). Neuroventilatory motor output from the isolated bullfrog brainstem was first characterized by Robert Schmidt to exclude breathing-related activity from his analysis of the neural correlates of vocalization (Schmidt, 1971). The preparation

was initially used for the study of vertebrate breathing by Heather McLean in John Remmer's laboratory, with the first publication coming from Richard Kinkead in William Milsom's laboratory (Kinkead and Milsom, 1994; McLean et al., 1995). In the time since those initial studies, the preparation has been used to study central mechanisms underlying vertebrate respiratory rhythmogenesis and chemosensitivity by many others (Galante et al., 1996; Gdovin et al., 1999; Harris et al., 2002; Milsom et al., 1999; Straus, 2000; Taylor et al., 2003a, 2003b; Torgerson et al., 2001).

The central hypercapnic response has been characterized throughout bullfrog development. Much like mammals, bullfrogs increase their lung burst frequency in response to hypercapnia (Fournier et al., 2007; Taylor et al., 2003a, 2003b; Torgerson et al., 1997). The hypercapnic response has been characterized as a 3-fold increase in the lung burst frequency, and this response is consistent across tadpole development (Taylor et al., 2003a, 2003b). At the receptor and neuroventilatory levels, the serotonergic, GABAergic, and cholinergic systems of the bullfrog are functionally similar to mammals (Broch et al., 2002; Brundage et al., 2010a, 2010b; Dickinson et al., 1988; Fournier et al., 2007; Shen et al., 1994). This suggests that the direct effects of nicotine or ethanol exposure are also comparable between bullfrogs and mammals, and when taken in concert with the advantages listed above

makes the bullfrog-tadpole model ideal for investigating the neuroventilatory effects of neuroteratogen exposure on central respiratory CO_2 chemosensitivity.

CHAPTER 1:

Central CO_2 chemosensitivity in tadpoles: Impairment and the role of serotonin[1]

Abstract

Chronic nicotine or ethanol exposure eliminates the hypercapnic response in the isolated tadpole brainstem preparation, and it is our hypothesis that this impairment is mediated by 5-HT_{1A} receptors (5HT-$_{1A}$Rs). Previous studies have shown that 8-OH-DPAT, a 5-HT_{1A}R agonist, elicits an increase in lung burst frequency exhibited by isolated tadpole brainstems. Here we show that, following nicotine or ethanol exposure, 8-OH-DPAT fails to increase lung burst frequencies. In addition to electrophysiological evidence, the current investigation made use of immunofluorescent imaging to measure general 5-HT_{1A}R and TrpOH levels. Immunofluorescent data showed a decrease in both measures for ethanol-exposed animals but no change in either following nicotine exposure. Taken together, these data provide evidence that the ethanol- and nicotine-induced impai[1]rment of the hypercapnic response is

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mediated by 5-HT_{1A}Rs and that the physiological relevant changes are due to changes to specific neurons rather than wide-spread changes.

1.1 Introduction

Breathing is largely an autonomic process controlled by neural circuitry in the brainstem. The breathing-control circuit comprises rhythmogenic neurons that connect, via interneurons, to motorneurons, which drive the breathing pump muscles. The circuit receives modulatory input that shapes the pattern of breathing and makes it responsive to changes in metabolism. Breathing rhythm is the result of many neurons, in multiple brainstem locations, interacting by their release and binding of numerous types of neurotransmitters and neuromodulators.

A subset of respiratory neurons are responsible for integration of the CO_2/H^+ stimulus into the activity of brainstem circuits, making breathing responsive to internal pH. Brainstem neurons of multiple neurotransmitter phenotypes have been implicated in CO_2 sensitivity. CO_2 sensitivity is decreased by interference with retrotrapezoid nucleus glutamatergic (Guyenet et al., 2009), locus coeruleus noradrenergic (Biancardi et al., 2008), ventro-medullary neurokinin-1 receptor-expressing (Akilesh et al., 1997) and raphe serotonergic (Hodges et al., 2004a, 2004b; Richerson et al., 2005) neurons in mammals. Additionally, Richerson has hypothesized that medullary serotonin (or 5-HT) neurons are key to CO_2 chemosensitivity due to their suitability to integrate synaptic input from multiple breathing-related sites as well as

their proximity to medullary arterial blood vessels (Richerson et al., 2005).

The isolated bullfrog brainstem offers numerous advantages for investigating the neural control of breathing. This experimental preparation provides, at any stage of development, the intact and functional central circuit that controls breathing and allows long-term investigation of central respiratory CO_2 responses (hypercapnic responses) under physiologically relevant conditions (Gdovin et al., 1999; Taylor et al., 2003a, 2003b). The frog hypercapnic response, a 3-fold increase in the frequency of lung-related neural activity, is consistent across tadpole development (Taylor et al., 2003b) unless impaired through chronic exposure to nicotine or ethanol (Brundage and Taylor, 2009, 2010; Taylor et al., 2008). Tadpoles thus provide an ideal opportunity to investigate the role of 5-HT in the hypercapnic response by comparing the effects of manipulating 5-HT signaling manipulation in hypercapnic-response-intact and hypercapnic-response-impaired animals.

While there is currently no defined genetic homology among amphibians and mammals with respect to either ontogeny or neural control of breathing, functional homologies exist and the relatedness of breathing in these two groups of vertebrates is accepted by respiratory physiologists. We expect that elucidating neural mechanisms of

breathing control in frogs will inform on similar processes in other vertebrates, even humans and their breathing-related pathologies. Thus, we were motivated to investigate whether tadpole brainstems with nicotine- and ethanol-induced impairment of CO_2 sensitivity have altered 5-HT signaling. It is our hypothesis that this is the case, and more specifically that the nicotine- and ethanol-induced impairment of CO_2 sensitivity includes a 5-HT_{1A}-dependent mechanism.

1.2 Methods

1.2.1 Animals

Experiments were performed on 40 early-stage (< stage 15) bullfrog tadpoles, *Lithobates catesbeianus*, obtained from a commercial supplier (Sullivan Co. Inc., www.researchamphibians.com). Tadpole stages were determined using anatomical features as described by Taylor and Kollros (1946). Animals were kept in aquaria filled with 12 L of dechlorinated tap water, maintained at room temperature and fed fish food daily. Toxin treatment groups were held in aquaria that contained either 18 μg/L nicotine or 0.1 % ethanol. The concentrations of nicotine and ethanol were consistent with those that impaired CO_2 sensitivity in previous studies using bullfrog tadpoles (Brundage and Taylor, 2009, 2010; Brundage et al., 2010b; Taylor et al., 2008). The concentration of nicotine was based on that found in the body fluids of average smokers

(Moyer et al., 2002). The ethanol concentration varied from 0.06 to 0.12 g/dL due to its volatilization from the aquarium water and was equivalent to 0.75-1.5 times the 0.08 g/dL blood alcohol content that is the legal limit for driving in many western countries. Animals were exposed to nicotine or ethanol for three or ten weeks, respectively, because these exposure durations have been repeatedly shown to reliably impair the central ventilatory responses to hypercapnia in early-stage tadpoles (Brundage and Taylor, 2009; Brundage et al., 2010a). All animal care and experimental procedures were carried out under Institutional Animal Care and Use Committee approval (UAF assurance # 04-07).

1.2.2 In Vitro Brainstem Preparation

Tadpoles were anesthetized by immersion in a cold (4 ^{o}C), 1 % solution of tricaine methane-sulfonate (MS222; Sigma, www.sigmaaldrich.com) in dechlorinated tap water buffered to pH 7.8 with $NaHCO_3$. Animals were judged to be adequately anesthetized when unresponsive to a tail pinch. Following anesthesia, a razor blade was used to remove the front of the head rostral to the nares and the back of the body, hind limbs, and tail.

Under a dissecting microscope the dorsal cranium was removed, the animal was decerebrated rostral to the diencephalon, and the fourth ventricle was opened by removing the choroid plexus. A section of brain

beginning immediately rostral to the optic tectum and ending caudal to the brachial nerve was removed. This section, principally the medulla, was further prepared by removal of the dura mater and trimming the ends of the cranial nerves. During the dissection, all exposed tissues were superfused with chilled artificial cerebrospinal fluid (aCSF), a solution that contained (in mM) 104 NaCl, 4 KCl, 1.40 $MgCl_2$, 10 d-glucose, 25 $NaHCO_3$, and 2.4 $CaCl_2$, which was equilibrated with 98.5 % O_2 and 1.5 % CO_2.

The brainstem was transferred to a 0.5-ml flow-through recording chamber and held ventral side up between two pieces of coarse nylon mesh. The chamber was supplied with aCSF flowing rostral to caudal over the brainstem at 5 ml/min. Depending upon the treatment desired, the aCSF was equilibrated with either 1.5 % (normocapnia) or 5.0 % (hypercapnia) CO_2, balance O_2. Both the chamber and aCSF were maintained at room temperature (23 ^{o}C) throughout the experiment.

1.2.3 Electrophysiology

Suction electrodes were constructed using 1-mm-diameter pulled-glass capillaries broken to tip diameters of 30-60 μm and smoothed with an emory file. Using light suction supplied by a syringe, roots of the facial and hypoglossal nerves were drawn into the suction electrodes. Whole-nerve output was captured by the electrode, amplified (100X DAM

50 amplifier, World Precision Instruments, www.wpiinc.com; and 1000X model 1700 four-channel amplifier, A-M Systems, www.a-msystems.com) and filtered (100 Hz high pass, 1 kHz low pass by the model 1700 amplifier). The amplified and filtered outputs were archived by a data acquisition system (Powerlab, AD Instruments, www.adinstruments.com), which sampled at 1 kHz and simultaneously recorded whole-nerve output and integrated neurograms (full wave rectified and averaged over 200 ms).

1.2.4 Experimental Protocol

Following placement in the recording chamber, brainstems were superfused with normocapnic aCSF and allowed to stabilize for 1 h prior to initiating the experimental protocol. Each experiment consisted of recording neural activity during 30 min of normocapnic baseline conditions (BL), 30 min of hypercapnic challenge (HC), 1 h of normocapnic recovery, 30 min of 8-OH-DPAT exposure (DPAT), 30 min of concomitant 8-OH-DPAT and hypercapnic exposure (HC/DPAT), and a final normocapnic washout to confirm that activity returned toward baseline levels. 8-OH-DPAT (8-hydroxy-N,N-dipropyl-2-aminotetralin; SIGMA, www.sigmaaldrich.com) is an agonist of medullary 5-HT_{1A} receptors. 5-HT_{1A} receptors are presynaptically located on serotonin neurons and act as inhibitory autoreceptors, their activation results in

hyperpolarization, decreased serotonin neuron firing, and reduced neurotransmitter release (Hjorth and Sharp, 1991; McCall et al., 1989). 8-OH-DPAT is commonly used in studies of the control of breathing to inhibit transmitter release by 5-HT neurons (Messier et al., 2004; Taylor et al., 2005). Two doses of 8-OH-DPAT were used, 0.5 and 5.0 μm. These doses have been previously shown to cause a significant increase in lung burst frequency (Kinkead et al., 2002). The 8-OH-DPAT/hypercapnia protocols were applied to brainstems isolated from both ethanol- and nicotine-treated animals. Thus, ethanol-treated animals at 0.5 μm (n = 5), nicotine-treated animals at 0.5 μm (n = 6), ethanol-treated animals at 5.0 μm (n = 5), and nicotine-treated animals at 5.0 μm (n = 8) were used.

Brainstems were also isolated from animals that had not been exposed to ethanol or nicotine in their aquarium water. After the 1-h stabilization period, whole-nerve output was recorded during 30 min of normocapnic baseline, 30 min of exposure to either 0.5 μm (n = 6) or 5.0 μm (n = 6) 8-OH-DPAT, and finally during a normocapnic washout. Another subset of control animals was used to confirm the concomitant effect of hypercapnia and 5.0 μm 8-OH-DPAT on activity of the respiratory neural circuit. After the 1-h stabilization period, whole-nerve output was recorded during 30 min of hypercapnia, 1-h normocapnic recovery, 30 min of 5.0 μm 8-OH-DPAT exposure, 30 min of concomitant hypercapnia and 8-OH-DPAT exposure (n =10).

1.2.5 Immunofluorescent Imaging of Serotonin Neurons

Brainstem preparations, which included tissue from the optic tectum, medulla, cerebellum and anterior portion of the spinal cord, were isolated from 8 early-stage tadpoles according to the protocol described above. Following electrophysiological experimentation, brainstems were fixed in 4 % paraformaldehyde in PBS for 24 h and 30 % glucose for 48 h. Brainstems were then frozen in optimum cutting temperature (OCT) compound (Fischer, www.fishersci.com).

Brainstem slices, 16-µm thick, located between cranial nerves (CN) 8 and 10 were mounted on gelatin-coated slides and stored at -80 ^{o}C until staining.

Slides with mounted brainstem slices were washed 3 times for 10 min in PBS. Slides were incubated for 2 h with blocker (3 % Triton-X and 5 % donkey serum in PBS) and for 48 h with the primary antibody solution at 4 ^{o}C. The primary antibodies were rabbit anti-tryptophan hydroxylase (TrpOH; 1:1000; Invitrogen Corp., www.invitrogen.com) and guinea pig anti-5HT$_{1A}$ receptor antibody (1:10,000; Invitrogen Corp., www.invitrogen.com). TrpOH, tryptophan hydroxylase, is the rate-limiting enzyme in serotonin production and is only expressed in serotonergic neurons. Thus, 5-HT$_{1A}$ receptors and TrpOH are both markers for serotonergic neurons. Following primary antibody exposure,

slides were rinsed twice for 5 min and once for 1 h in PBS. Slides were incubated for 1 h with secondary antibody solution containing anti-rabbit Alexa 488 (1:700; Invitrogen Corp., www.invitrogen.com) and anti-guinea pig Alexa 546 (1:200; Invitrogen Corp., www.invitrogen.com). Following the secondary incubation, slides were rinsed with PBS 6 times for 5 min intervals and stored at 4 ^{o}C while drying prior to being hydrated with Vectashield (Vector Laboratories, Inc., www.vectorlabs.com) and cover-slipped.

Slices were viewed and images acquired at 4X magnification with a numerical aperture of 0.20. Images of cross-sectional slices from respiratory-related areas of the brainstem, the raphe regions, and the raphe obscurus (RO) were acquired at 10x magnification, with a numerical aperture of 0.30. Fluorescence intensity due to 5-HT_{1A} receptor or TrpOH staining, measured in gray values, was acquired using NIS-Elements software (Nikon; www.nis-elements.com/).

1.2.6 Data analysis and statistics

1.2.6.1Electrophysiology

Bursts in the neurograms were designated as either a gill or lung activity on the basis of amplitude of the integrated nerve activity and presence or absence of coincident firing by the facial and hypoglossal nerves as previously described (Gdovin et al., 1999).

Gill bursts had lower amplitude on the facial nerve than lung bursts and little or no coincident burst activity in the hypoglossal nerve. Lung bursts had higher amplitude in the facial nerve and were coincident in the hypoglossal nerves. Frequencies of lung bursts were quantified over the last 3 min of each experimental condition (baseline normocapnia, hypercapnia, 8-OH-DPAT + normocapnia, and 8-OH-DPAT + hypercapnia, which are abbreviated BL, HC, DPAT, and HC/DPAT respectively). Mean values for the burst frequencies were compared using one-way, repeated-measures analysis of variance (RM-ANOVA; SigmaStat, www.systat.com). When RM-ANOVA indicated that significant differences existed, multiple comparisons were made using the Holm-Sidak multiple comparison test. All values are reported in the text and figures as mean ± standard error.

1.2.6.2 Immunofluorescence

Immunofluorescence intensity from 3-6 medullary slices from each animal were averaged and these values were used in an ANOVA to compare between neuroteratogen-exposed and unexposed animals.

1.3 Results

We investigated the effect of blocking 5-HT signaling with 8-OH-DPAT on the central control of lung ventilation in early-stage tadpoles. This was done in both control animals and those whose central ventilatory CO_2 response had been impaired by exposure to ethanol or nicotine. Isolated brainstems from control and toxin-exposed tadpoles were treated with a dose of 5.0 μm 8-OH-DPAT. This dose was selected based on the results of initial experiments using doses of 0.5 and 5.0 μm in which only the 5.0 μm dose was found to be effective in modulating lung burst frequency (Fig. 1.1) and the bullfrog central hypercapnic response (Fig. 1.2). This finding is similar to the finding of Kinkead et al., (2002) who reported significant effects at both 0.5 and 5.0 μm. In our hands 0.5 μm was not an effective dose for augmenting lung burst frequency. This disparity is most likely due to the Kinkead et al.'s use of a high-affinity, single-enatiomer version of 8-OH-DPAT while this study used a lower affinity racemic mixture.

1.3.1 5-HT Signaling Modulates Lung Burst Frequency During Normocapnia but not the Hypercapnic Response

In the initial 8-OH-DPAT dose validation (see Fig 1.1), the brainstems exposed to 0.5 μm 8-OH-DPAT failed to display a change in lung burst frequency ($p > 0.05$; $n = 6$). This contrasts with brainstems

from the initial 5.0 μm 8-OH-DPAT experiments where normalized lung burst frequency during 8-OH-DPAT exposure was 8.5 ± 3.5 bursts/min, a significant increase in lung frequency ($p = 0.0137$).

5.0 μm 8-OH-DPAT was the only dose used to determine the role of 5-HT signaling in the central hypercapnic response. In this data set absolute lung burst frequencies were as follows: BL 0.3 ± 0.1, HC 2.4 ± 0.3, 5.0 μm DPAT 1.4 ± 0.3, and HC/5.0 μm DPAT 2.0 ± 0.4 bursts/min. Analysis by one-way repeated-measures ANOVA indicated a significant difference between BL and HC ($p < 0.001$), BL and 5 μm 8-OH-DPAT ($p = 0.043$), and BL and HC/5 μm DPAT($p = 0.002$), but failed to detect significant differences between any other comparisons ($p \geq 0.09$). See Fig 1.2.

1.3.2 5-HT Signaling Fails to Modulate Lung Burst Frequency in Nicotine- or Ethanol-Exposed Tadpoles

Mean lung burst frequencies for toxin-exposed animals, expressed as absolute frequencies, are shown in Figs. 1.3 and 1.4.

In isolated brainstems from the 3-wk nicotine-exposed animals, lung burst frequency during BL was 1.1 ± 0.2 bursts/min and 5.0 μm 8-OH-DPAT did not significantly affect this frequency ($p > 0.05$; $n = 8$). 5.0 μm 8-OH-DPAT also failed to increase lung burst frequency in the 10-wk EtOH animals where lung burst frequency during BL was 1.1 ± 0.5

bursts/min ($p > 0.05$; $n = 5$). Analysis by one-way repeated-measures ANOVA indicated no significant differences between any pairwise comparison in either neuroteratogen-exposed group ($p > 0.324$).

1.3.3 Chronic Ethanol Exposure Greatly Reduces Densities of 5-HT_{1A} Receptors and Tryptophan Hydroxylase.

Immunofluorescence levels for control and toxin-exposed animals are displayed in Figs 1.5 and 1.6.

The density of 5-HT_{1A} receptors and TrpOH in respiratory-related areas of the tadpole medulla were quantified by immunofluorescent imaging. Immunofluorescence grey values from brainstem slices located between cranial nerves (CN) 8 and 10 in control animals for 5-HT_{1A} receptors were 529 ± 149 ($n = 5$) and values for TrpOH levels were 233 ± 25 ($n = 5$).

In brainstem slices from 3-wk nicotine-exposed animals, immunofluorescence grey values in the same area were not significantly different from controls for either 5-HT_{1A} receptor densities (264 ± 25 ; $n = 4$) or TrpOH levels (129 ± 51; $n = 4$).

Immunofluorescence grey values in 10-wk ethanol-exposed animals for 5-HT_{1A} receptors and TrpOH were 86 ± 4 and 46 ± 9 respectively. When compared to control animals, these data indicate an 84 % decrease in 5-HT_{1A} receptor densities and an 80 % decrease in

TrpOH densities in the ethanol-exposed animals (n = 6, p = 0.0027 and n = 5, p = 0.0007, respectively.

1.4 Discussion

It has been previously shown that chronic exposure to nicotine or ethanol eliminate the normally robust increase in lung burst frequency, the hypercapnic response, elicited from isolated tadpole brainstems by elevated CO_2 levels (Brundage and Taylor, 2009; Taylor et al., 2008). Our results show that the lung burst frequencies of brainstems from animals chronically exposed to nicotine or ethanol fail to respond to 5.0 μM 8-OH-DPAT under either normocapnic or hypercapnic conditions, consistent with our hypothesis that 5-HT plays a role in hypercapnic responses.

Although brainstems from control animals showed an increase in lung burst frequencies in response to either hypercapnia or 5.0 μM 8-OH-DPAT during normocapnia, there is no additional increase in lung burst frequency with concomitant 5.0 μM 8-OH-DPAT exposure and hypercapnia. A possible explanation for this is that individual exposure to either 5 % CO_2 or 5.0 μM DPAT saturates the available physiological response of the isolated brainstem such that concomitant exposure cannot elicit a further increase.

Brainstems isolated from ethanol- or nicotine-exposed animals failed to show a lung burst frequency response to hypercapnic or 5.0 μM 8-OH-DPAT exposure. That these animals do not respond to concomitant exposure to these conditions is noteworthy insomuch as it provides support for our hypothesis that the toxin-induced abolishment of the hypercapnic response is a 5-HT_{1A} receptor-mediated process. If the nicotine- or ethanol-induced abolishment of the hypercapnic response is a 5-HT_{1A} receptor-dependent process, then one would expect control animals to respond differently to a 5-HT_{1A} receptor ligand, such as 8-OH-DPAT, than would a toxin-exposed animal.

Immunofluorescence data collected from brainstem slices of 10-wk ethanol-exposed animals clearly shows a decrease in both 5-HT_{1A} receptors and TrpOH levels in the medulla between cranial nerves 8 and 10. In contrast to this, 3-wk nicotine-exposed animals did not display a statistically significant difference in immunofluorescence for either of these proteins.

Taken together these electrophysiological and immunofluorescent data suggest that the deleterious effects of ethanol or nicotine exposure, which leads to impairment of the hypercapnic response, include a failure of serotonergic signaling and that this failure is not simply the reflection of a global reduction in serotonin levels. Rather, the implication is that key serotonergic neurons, or populations of neurons, are negatively

impacted and the ablation of the normal physiological functioning of these specific neurons leads to the loss of hypercapnic sensitivity.

Figures

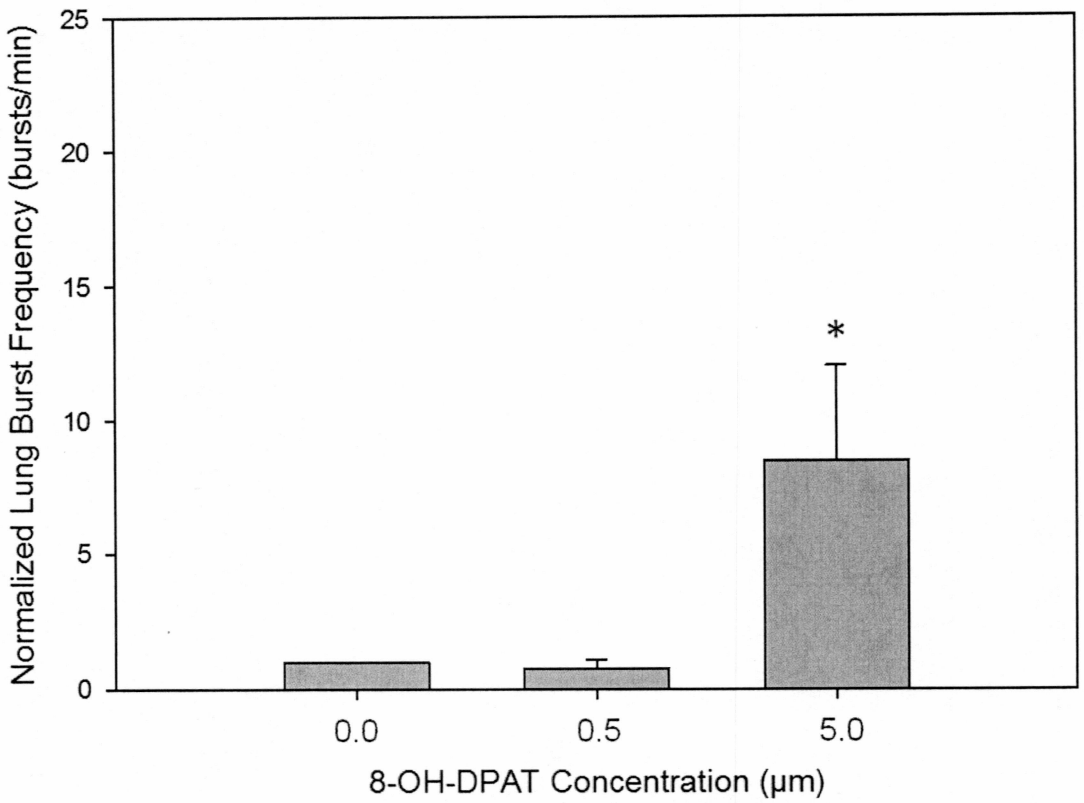

Figure 1.1 Initial 8-OH-DPAT dose response. Normalized (relative to baseline measurements) lung burst frequencies exhibited by brainstems isolated from control tadpoles. In the 0.5 µm experiments, lung burst frequency during baseline was 1.8 ± 0.1 bursts/min and 0.5 µm 8-OH-DPAT did not significantly affect this frequency ($p > 0.05$; DPAT:BL ratio of lung burst frequency was 0.7 ± 0.3). In the 5.0 µm experiments lung burst during baseline was 1.9 ± 0.6 bursts/min and 5.0 µm 8-OH-DPAT significantly increased this frequency ($p = 0.0137$; DPAT:BL ratio of lung burst frequency was 8.5 ± 3.5). Data are mean ± SE; asterisk indicates a significant difference in lung burst frequency relative to control conditions.

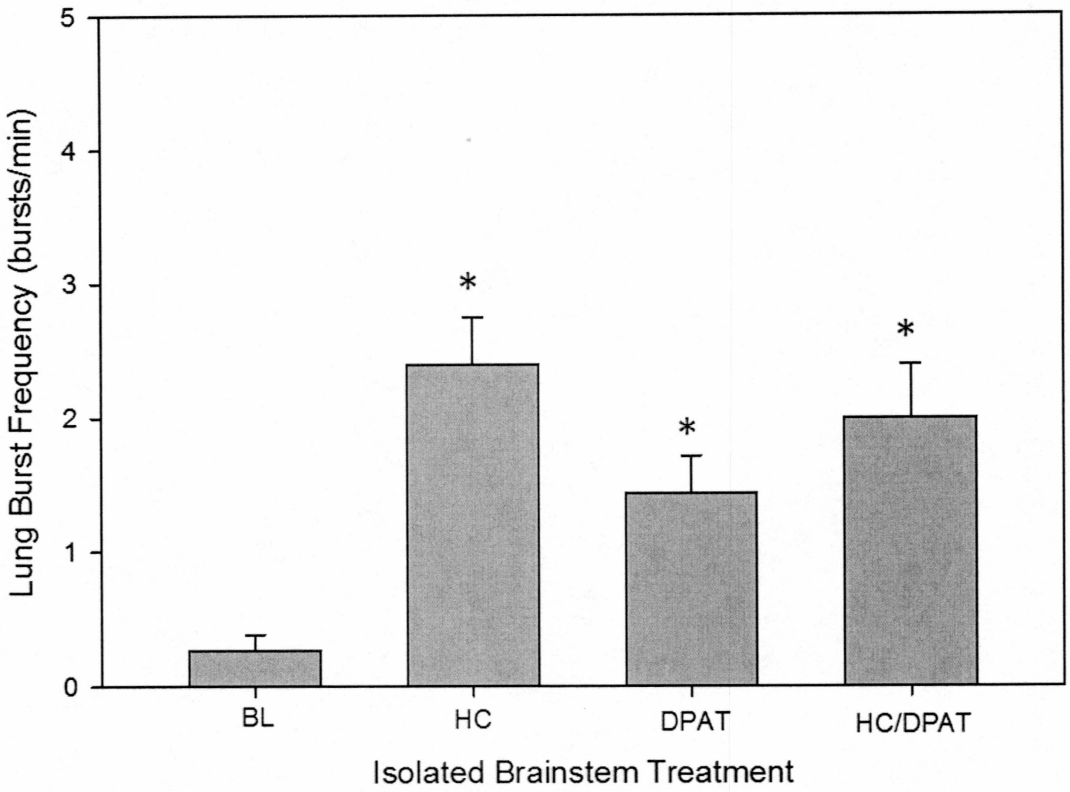

Figure 1.2 Secondary 8-OH-DPAT dose response. Lung burst frequencies exhibited by brainstems isolated from control tadpoles. Lung burst frequency during baseline normocapnia (BL) was 0.3 ± 0.1, during hypercapnia (HC) was 2.4 ± 0.1, during normocapnia plus 8-OH-DPAT (DPAT) was 1.4 ± 0.3, and during hypercapnia plus 8-OH-DPAT (HC/DPAT) was 2.0 ± 0.4 bursts/min. Repeated Measures ANOVA indicated significant differences between BL and HC, DPAT, and HC/DPAT ($p < 0.001$, $p = 0.043$, $p = 0.002$, respectively), but there were no significant differences among any other comparisons ($p < 0.093$ in all cases). Data are mean ± SE; asterisks indicate significant differences in lung bursts frequencies relative to baseline conditions.

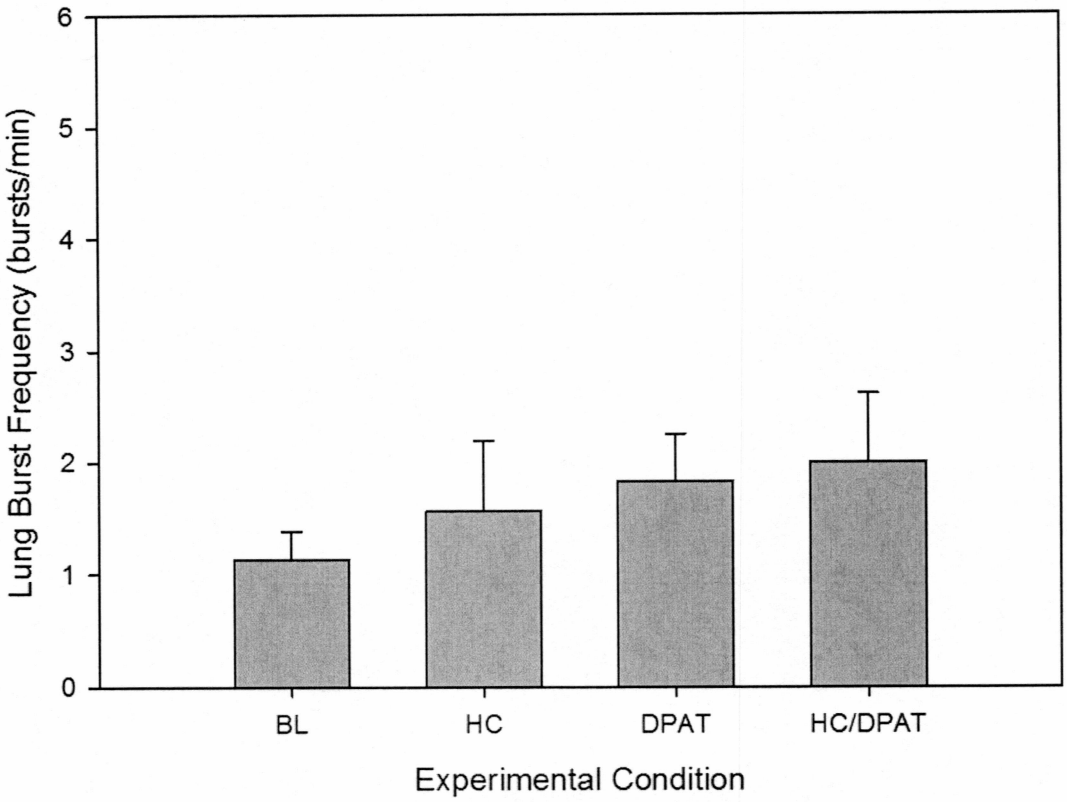

Figure 1.3 3-week nicotine-exposed lung burst frequencies. Lung burst frequency from isolated brainstems during baseline normocapnia (BL) was 1.4 ± 0.2 bursts/min and neither hypercapnia (HC), 5.0 μm 8-OH-DPAT (DPAT), nor HC plus DPAT significantly affectd this frequency ($p > 0.05$). Data are mean ± SE.

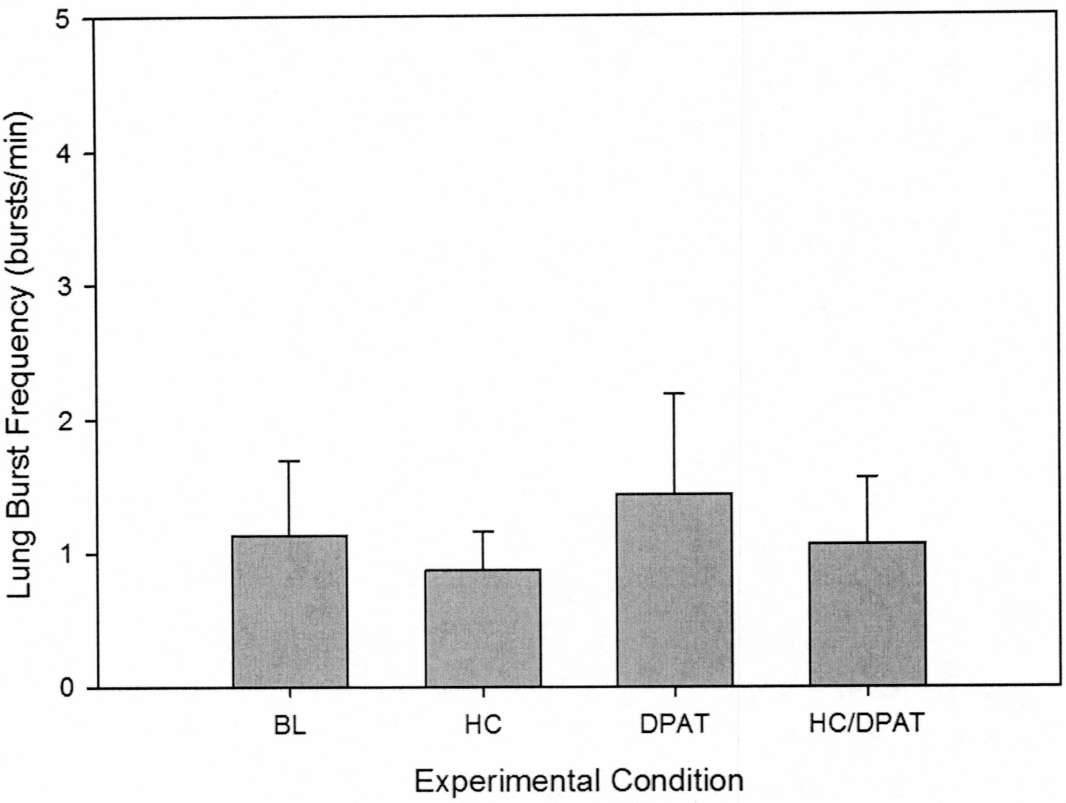

Figure 1.4 10-week ethanol-exposed lung burst frequencies. Lung burst frequencies exhibited by brainstems isolated from 10-wk, ethanol-exposed tadpoles. Lung burst frequency during baseline normocapnia (BL) was 1.1 ± 0.6 bursts/min and neither hypercapnia (HC), 5.0 μm 8-OH-DPAT (DPAT), nor HC plus DPAT significantly affectd this frequency ($p > 0.05$). Data are mean ± SE.

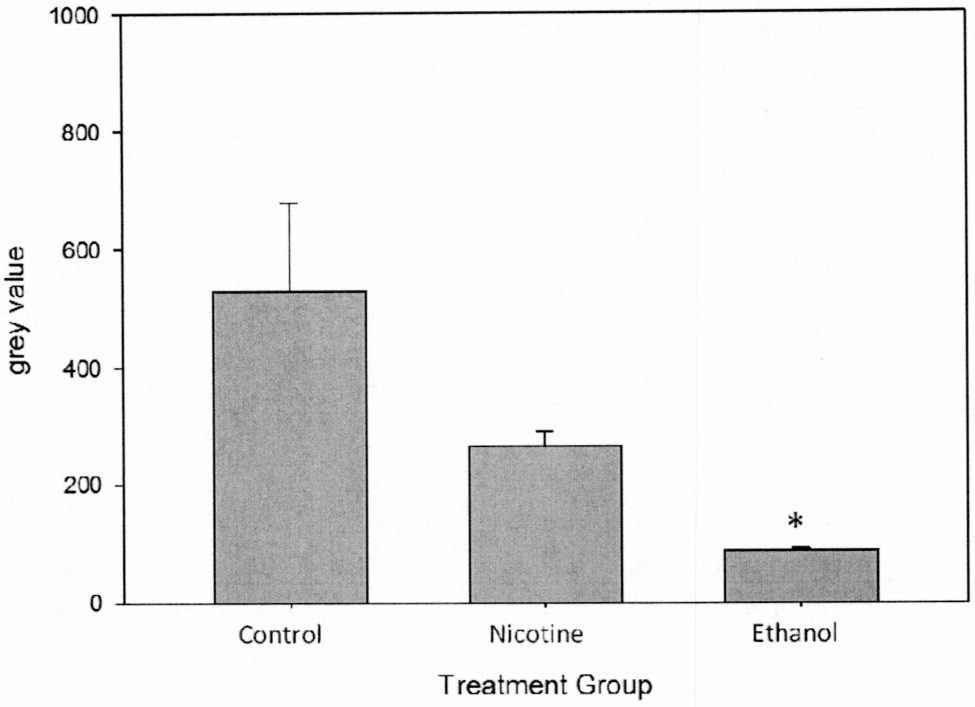

Figure 1.5 5-HT Immunofluorescence values. Fluorescence intensity (measured in gray values) of brainstem slices in which 5-HT_{1A} receptors were immunofluorescently labeled. Slices included the entire cross-section of the brainstem between cranial nerves 5 and 10 isolated from tadpoles unexposed to neuroteratogens (control), exposed to nicotine for 3 wk, or exposed to ethanol for 10 wk. 10-wk ethanol exposed resulted in a significant reduction in 5-HT_{1A}-receptor staining (p=.0027). Data are mean ± SE, asterisk indicates significant difference in lung bursts frequency relative to baseline conditions.

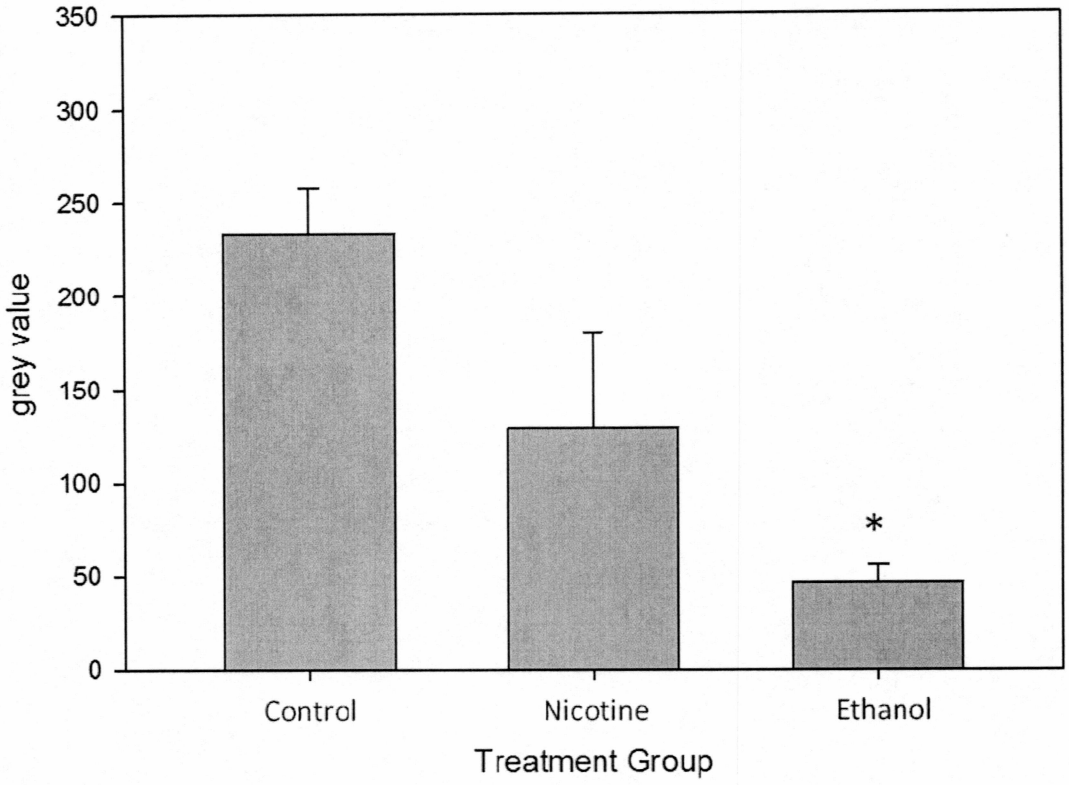

Figure 1.6 TrpOH Immunofluorescence values. Fluorescence intensity (measured in gray values) of brainstem slices in which tryptophan hydroxylase was immunofluorescently labeled. Slices included the entire cross-section of the brainstem between cranial nerves 5 and 10 isolated from tadpoles unexposed to neuroteratogens (control), exposed to nicotine for 3 wk, or exposed to ethanol for 10 wk. 10-wk ethanol exposed resulted in a significant reduction in tryptophan hydroxylase staining ($p=.0007$). Data are mean ± SE, asterisk indicates significant difference in lung bursts frequency relative to baseline conditions.

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GENERAL CONCLUSIONS

That developmental exposure to ethanol or nicotine is deleterious to neural function is widely accepted. Of particular applicability to the current study is the recent characterization of the developmental consequences of ethanol or nicotine exposure to control of breathing in developing bullfrog tadpoles (Brundage and Taylor, 2009; Brundage et al., 2010a, 2010b; Taylor et al., 2008). These studies showed that 10-wk exposure to 0.1 % ethanol or 3-wk exposure to 18 μg/L nicotine exposure impairs central responses to hypercapnia in early-stage bullfrog tadpoles (Brundage and Taylor, 2009; Taylor et al., 2008). The current study replicated those findings and attempted to elucidate a causative mechanism for the CO_2-induced impairment of respiratory drive. Our hypothesis, that the nicotine- and ethanol-induced impairment of CO_2 sensitivity caused by ethanol or nicotine exposure is mediated by a 5-HT_{1A}-dependent mechanism, was supported by electrophysiological evidence. Acute exposure to 5.0 μm 8-OH-DPAT elicited an 8-fold increase in lung burst frequencies in early-stage tadpoles and this effect is not observed following chronic ethanol or nicotine exposure of the tadpoles (see Chapter 1). As a 5-HT_{1A}-specific agonist, the effects of 8-OH-DPAT application would be expected to differ between animals with altered density or functionality of 5-HT_{1A} receptors. This

electrophysiological evidence is not a definitive confirmation of our hypothesis but is consistent with expected results.

Immunofluorescent staining data collected from brainstems isolated from tadpoles chronically exposed to nicotine or ethanol does not definitively support the hypothesis. Rather these data suggest that the deleterious effects of ethanol or nicotine exposure are a failure of serotonergic signaling on a neuronal rather than a global level. If toxin exposure had caused widespread change in 5-HT_{1A} receptor density we would have observed similar immunofluorescent trends from both ethanol- and nicotine-exposed animals. What was observed was that ethanol exposure greatly reduced 5-HT_{1A} receptor densities and TrpOH levels, while nicotine exposure had no effect on either measure. The similar electrophysiological responses to elevated CO_2 exhibited by both toxin-exposed groups showed that the hypercapnic response had indeed been ablated despite the difference in immunofluorescent labeling of protein markers of serotonergic neurons. Taken together these data imply that key serotonergic neurons, or populations of neurons, are negatively impacted by ethanol or nicotine exposure.

To continue this line of investigation, future studies should make use of 5-HT_{1A} antagonists and experimental methods such as focal perfusion or localized injections of 5-HT_{1A} receptor ligands. Furthermore, previous studies have determined the duration of recovery times needed

following chronic toxin exposure to reinstate the hypercapnic response. In early stage tadpoles, following 10-wk ethanol exposure 6 wk of recovery is required (Brundage and Taylor, 2010). This same study determined that 3 wk of recovery following 10-wk of nicotine exposure was sufficient to recover the hypercapnic response but this timeline would need to be substantiated for the 3wk exposure used in the current study. Comparing responses to 5-HT_{1A} receptor ligands, like 8-OH-DPAT, between brainstems from tadpoles that had recovered their CO_2-sensitivity and those that had never had their CO_2-sensitivity impaired will be of great interest. Similar responses to 5-HT_{1A} receptor ligands would further support the role of 5-HT signaling in the hypercapnic response and suggest that recovery was based on recovery of 5-HT signaling. If, however, the recovered brainstems, like toxin-impaired brainstems, were insensitive to 5-HT_{1A} receptor ligands, then recovery of central CO_2 sensitivity would indicate development of a new mechanism for signaling tissue CO2 status. Such findings would lead to an interesting and fruitful line of research, one likely to enhance our understanding of CO_2 sensitivity and recovery of neuronal function after cessation of toxin exposure.

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